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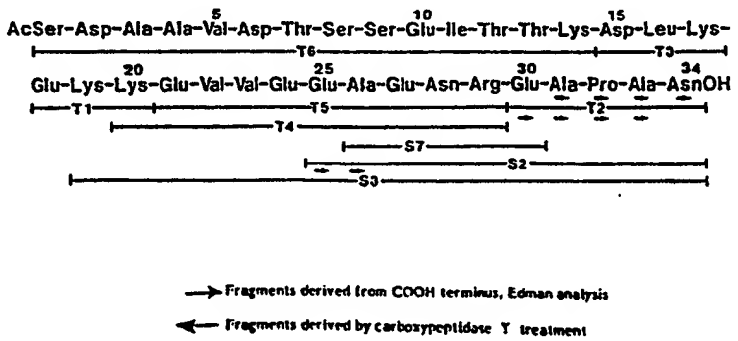
(54) **Thymosin alpha 11.**

(57) A polypeptide useful in the reconstitution of immune functions in thymic deprived or immunodeprived warm-blooded mammals of the formula

AcSer-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-Gly-Arg-Glu-Ala-Pro-Ala-AsnOh (I)

and pharmaceutically acceptable salts thereof as well as pharmaceutical compositions containing them.

FIG 2



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Thymosin α_{11}

Thymosin fraction 5, known for example from U.S. Patent 4,082,737, is a potent immunopotentiating preparation and can act to reconstitute immune functions in thymic deprived and/or immunodeprived individuals. Ongoing clinical trials with thymosin fraction 5 suggest that it is effective in increasing T cell numbers and normalizing immune function in children with thymic dependent primary immunodeficiency disease and can increase T cell numbers in immunodepressed cancer patients.

The first active peptide isolated and characterized from thymosin fraction 5 has been termed thymosin α_1. The isolation and characterization of this peptide is described for example in U.S. Patent 4,079,127. Synthesis of thymosin α_1 by solution and solid phase synthesis is described in U.S. Patent 4,148,788. Additionally the synthesis of thymosin α_1 by solution phase procedures is shown in U.S. Patent 4,116,951. Thymosin α_1 has been found to be one or more orders of magnitude more active than fraction 5 in several in vitro and in vivo assay systems designed to measure T cell differentiation and function and is currently in the clinic to determine its efficiacy in the treatment of immunodeficiency diseases, of immunodepressed cancer patients and in the prevention of opportunistic infections in immunosuppressed patients.

The present invention relates to the isolation and first complete structural determination of a new polypeptide (thymosin α_{11}) isolated from thymosin fraction 5, to pharmaceutically acceptable acid and base addition salts thereof and to pharmaceutical compositions containing this

compound or its salts. Thymosin α_{11} has been found to have the same qualitative and quantitative biological activity as thymosin α_1 in in vivo assay systems designed to measure T cell differentiation and function.

Thymosin α_{11} is a polypeptide of 35 amino acid residues, the first 28 of which are identical to thymosin α_1. Thymosin α_{11} has the following amino acid sequence:

AcSer-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-Gly-Arg-Glu-Ala-Pro-Ala-AsnOH

wherein Ac is an amino terminal acetyl group.

Thymosin α_{11} was isolated from calf thymus fraction 5 by a combination of preparative isoelectric focusing (see Hannappel et al., Proc. Natl. Acad. Sci. USA 79, 1708-1711 [1982]) and HPLC (see Stein and Moschera, Methods in Enzymology 79, 7-16 [1981]).

Thymosin fraction 5 prepared in accordance with known procedures, see e.g. U.S. 4,079,127, was electrofocused for 17 hours at a maximum current of 20mA and a maximum voltage of 1.1 kV. The gel bed was divided into 30 segments (fractions) with a stainless steel grid and peptides from each segment were eluted with 5 ml of water. The pH of each eluate was determined with a pH meter.

For analysis of the peptides in the eluates by HPLC, aliquots were lyophilized and dissolved in a small volume of buffer A (0.2M pyridine, 1.0M in formic acid). Elution was with buffer A and a linear gradient of 1-propanol.

In Figure 1 results are shown for the HPLC analysis of fractions 6 and 7. Peaks a, b, and c were identified as

des-(25-28)-thymosin α_1, thymosin α_1, and thymosin α_{11} respectively. Some additional thymosin α_1 was recovered from isoelectric focusing fractions 4 and 5 and a small quantity of thymosin α_{11} was also present in fraction 5.

Separation of peptides by HPLC was performed with an Ultrasphere ODS C18 column (5 µ, 4.6 x 250 mm, Altex Scientific) with a fluorescamine detection system as described by Stein and Moschera, supra.

The run shown in Figure 1 was derived from a 2 gram batch of calf thymosin fraction 5. The peptides in peak c of the HPLC purification step from isoelectric focusing fractions 6 and 7 were combined, lyophilized and purified by rechromatography on HPLC utilizing the methodology described for Figure 1. An aliquot (600 µg) was digested with 42.9 µg of TPCK-treated trypsin in 100 µl of 0.4M pyridine, pH 7.5. After 15 hours at 25°C the reaction mixture was lyophilized and the tryptic peptides were separated by HPLC using a gradient of acetonitrile (0 to 30 volume percent). Fractions (0.65 ml) were collected every minute. At 6-second intervals, 5 µl samples were diverted to the fluorescamine detector.

Six fragments were recovered and identified by their amino acid composition as summarized below in Table 1.

Table 1. Amino acid composition of peptides isolated from tryptic and S. aureus V8 protease digests of thymosin α_{11}

Residue	T1 (68)*	T2 (53)*	T3 (57)*	T4 (60)*	T5 (32)*	T6 (66)*	S2 (2.6)+	S3 (0.8)+	S7 (3.7)+
Asp		0.8	1.3	1.0	1.1	2.2	1.3	1.4	1.4
Thr						2.7			
Ser						2.7			
Glu	1.0	1.0		3.4	3.3	1.1	2.4	4.8	1.7
Gly				1.0	1.0		1.4	1.1	1.9
Ala		2.0		1.0	1.0	1.9	2.6	2.7	1.0
Val				1.9	1.5	1.1		1.0	
Ile						1.0			
Leu			0.8						
Lys	2.1		1.0	0.2		1.0		2.9	
Arg				1.0	1.1		1.0	1.0	0.5
Pro		1.3					nd**	nd**	nd**

Calculated based on assigning a value of 1.0 for the residue as underlined.

* Nanomoles recovered from a digest of 200 nanomoles of thymosin α_{11}.

+ Nanomoles recovered from a digest of 8.7 nanomoles of thymosin α_{11}.

** Not determined.

Peptides T6, T3 and T1 were identical to peptides derived from residues 1-14, 15-17 and 18-20, respectively, of thymosin α_1. Peptides T4 and T5 were similar in amino acid composition, differing only in the presence of lysine in peptide T4. Their composition indicated that they corresponded to residues 20-28 of thymosin α_1, plus glycine and arginine. Since peptide T5 did not contain lysine, it was concluded that arginine must be located at the COOH terminus of the peptide (see Figure 2). The tryptic digest contained an additional peptide fragment (T2) which was not present in tryptic digests of thymosin α_1. This new peptide fragment contained no lysine or arginine, and must therefore have arisen from the COOH-terminus of thymosin α_{11}.

Edmann degradation of tryptic peptide T2 yielded the sequence Glu-Ala-Pro-Ala-Asn-OH. This data is summarized in Table 2.

Table 2 Edman degradation of tryptic peptide T2

Step	Nanomoles of peptide recovered after each step of degradation	Subtractive method, amino acid composition of residual peptides*)					Recovered after hydrolysis of the anilinothiazolinone(nmol)**)	PTH amino acid identified+)
		Glx	Ala	Pro	Ala	Asx		
0	(35.3)***)	1.3	1.2	1.7	1.2	1.0		
1	28.8++)	0.1	1.1	1.4	1.1	1.0	Glu (6.9)	Glu
2	30.6++)	0.2	0.1	1.1	1.2	1.0	Ala (5.9)	Ala
3	36.7++)	0.4	0	0.6	1.2	1.0	Pro (2.6)	Pro
4	30.3++)	0.3	0	nd	0.4	1.0	Ala (2.8)	Ala

*) Amino acid compositions of an aliquot of thymosin α_{11} or of aliquots from the aqueous phase after each step of Edmann degradation. The results are presented as ratios to the quantity of aspartic acid. Half the total for alanine was arbitrarily assigned to each alanine residue in the sequence for the first 2 steps.

**) An aliquot for each anilinothiazolinone was removed before cyclization and hydrolyzed for amino acid analysis.

***) This quantity was used for the degradation procedure.

+) The PTH amino acids obtained at each step of the degradation were identified by HPLC.

++) Estimated from the results of amino acid analysis after acid hydrolysis.

Asparagine was recovered as the free amino acid after the fourth step of the Edmann procedure. Localization of peptide T2 at the COOH terminus of thymosin α_{11} was confirmed by digestion of the later with carboxypeptidase (Y) which released approximately one equivalent of asparagine, followed by alanine (2 equivalents) and proline (one equivalent). The location of arginine at position 30 was confirmed by the isolation of a major fragment containing arginine after digestion of thymosin α_{11} with S. aureus V8 protease (peptide S7 in Table 1). The amino acid composition of this peptide corresponded to that predicted for residues 26-31 of thymosin α_{11} including the last four residues of thymosin α_1, plus the first three amino acid residues, glycine, arginine and glutamic acid, found in the COOH-terminal extension of thymosin α_{11}. Smaller quantities of two of the fragments, whose amino acid composition corresponded to residues 19-35 (peptide S3) and 25-35 (peptide S2) of thymosin α_{11} were also isolated from the S. aureus protease digests (Table 1 and Figure 2). The results establish thymosin α_{11} as containing the thymosin α_1 sequence plus seven additional amino acids as the COOH-terminus.

The biological activity of thymosin α_1 can be determined by utilizing in vivo assays known in the art. Thus, for example, inbred strains of mice are known to vary in their susceptability to infection with C. albicans. Thus, mice of such strains as C_3 H/HeJ or CBA/CaJ are highly susceptible to infection, whereas mice of such strains as C_{57} Bl/10SNJ or C_{57}Bl/KsJ were highly resistant to challenge. Since resistance to infection with C. albicans is associated with cell-mediated processes, and therefore with T-lymphocytes, thymic hormones should have an effect on the host response. Thymosin fraction 5 and some peptides derived therefrom have been found to enhance maturation and replication of T-lymphocytes (Goldstein et al., Rec. Progress in Hormone Research 37, 369-415 [1981])

and accordingly should influence the resistance of a susceptible murine strain, such as C_3H/HeJ, to infection with C. albicans.

Thymosin fraction 5, thymosin α_1 or α_{11} was injected daily i.p. in graded doses into three different groups of mice, beginning two days before intravenous challenge with 4×10^4 cells of Candida albicans. In comparison with control mice, all three thymic derivatives provided protection. The results are summarized in Table 3 below.

Table 3. Effect of thymosin fraction 5 and thymic peptides on the growth of Candida albicans in C_3H/HeJ mice

Thymosin fraction 5		Thymosin α_1		Thymosin α_{11}	
Dose ng/mouse	C. albicans cell count*	Dose ng/mouse	C. albicans cell count*	Dose ng/mouse	C. albicans cell count*
2560	8500	80	5870	80	4200
5120	440	160	190	160	510
10240	320	320	780	320	320
20480	1600	640	1410	640	1260

Mice were treated daily with the indicated doses of thymosin fraction 5, thymosin α_1 or thymosin α_{11} and challenged with 4×10^4 cells of C. albicans two days after the start of treatment

*Three mice from each set were sacrificed on days 7, 14 and 21 after infection and the values represent the average number of organisms in the left kidneys of the nine mice in each set.

The polypeptides, thymosin α_1 and thymosin α_{11}, were approximately equal in potency, being most active in daily doses of 160-320 ng per mouse i.p. Since the optimum dose for fraction 5 was 5-10 µg, the peptides were about 30 times more potent than fraction 5 in their ability to induce resistance to infection with C. albicans.

Thus, thymosin α_{11}, in analogy to thymosin α_1, can be used as therapeutical agent, especially as an agent useful in the reconstitution of immune functions in thymic deprived or immunodeprived warm-blooded mammals. It may be administered in the form of pharmaceutical compositions, i.e. in mixture with a suitable pharmaceutically acceptable carrier and, if desired, further adjuvants, to warm-blooded mammals by parenteral application either intravenously, subcutaneously or intramuscularly. The compound is a potent immunopotentiating agent with a daily dosage in the range of about 1 to 100 µg/kg of body weight for intravenous administration. Obviously the required dosage will vary with the particular condition being treated, the severity of the condition and duration of the treatment. A suitable dosage form for pharmaceutical use is 1 mg of lyophylized thymosin α_{11} per vial to be reconstituted prior to use by the addition of sterile water or saline.

Also included within the scope of the present invention are the pharmaceutically acceptable salts of thymosin α_{11} such as the sodium or potassium salts or salts with strong organic bases such as guanidine. In addition, the counter ions of these cations as well as of lysine residues such as the chloride, bromide, sulphate, phosphate, maleate, acetate, citrate, benzoate, succininate, ascorbate and the like, may be included in the preparation.

It is also within the scope of the present invention to modify the sequence of thymosin α_{11} by single amino acid changes or by derivatizing the carboxy terminus by

ester or amide formation. Also within the scope of the invention is desacetyl thymosin α_{11} which can be produced microbially, using recombinant DNA techniques, e.g. a synthetic gene which is introduced into a suitable microbial expression vehicle. After transformation with such an expression vehicle microorganisms or mammalian cells will be able to express desacetyl thymosin α_{11} under suitable conditions. Desacetylthymosin α_{11} would have the same biological activity as thymosin α_{11} by analogy to the relationship between thymosin α_1 and desacetylthymosin α_1.

CLAIMS:

1. Thymosin α_{11}, a polypeptide of the sequence

AcSer-Asp-Ala-Ala-Val-Asp-Thr-Ser-Ser-Glu-Ile-Thr-Thr-Lys-Asp-Leu-Lys-Glu-Lys-Lys-Glu-Val-Val-Glu-Glu-Ala-Glu-Asn-Gly-Arg-Glu-Ala-Pro-Ala-AsnOH (I)

and pharmaceutically acceptable acid and base addition salts thereof.

2. The peptide of formula I as claimed in claim 1 essentially free of other thymic polypeptides.

3. A compound as claimed in claim 1 or claim 2 for use as therapeutical agent.

4. A compound as claimed in claim 1 or claim 2 for use as an agent reconstituting immune functions in thymic deprived or immunodeprived warm-blooded mammals.

5. Pharmaceutical compositions containing a compound as claimed in claim 1 or claim 2 and a suitable, pharmaceutically acceptable carrier.

6. Compositions useful in the reconstitution of immune functions in thymic deprived or immunodeprived warm-blooded mammals containing a compound as claimed in claim 1 or claim 2 and a suitable, pharmaceutically acceptable carrier.

***

Figure 1

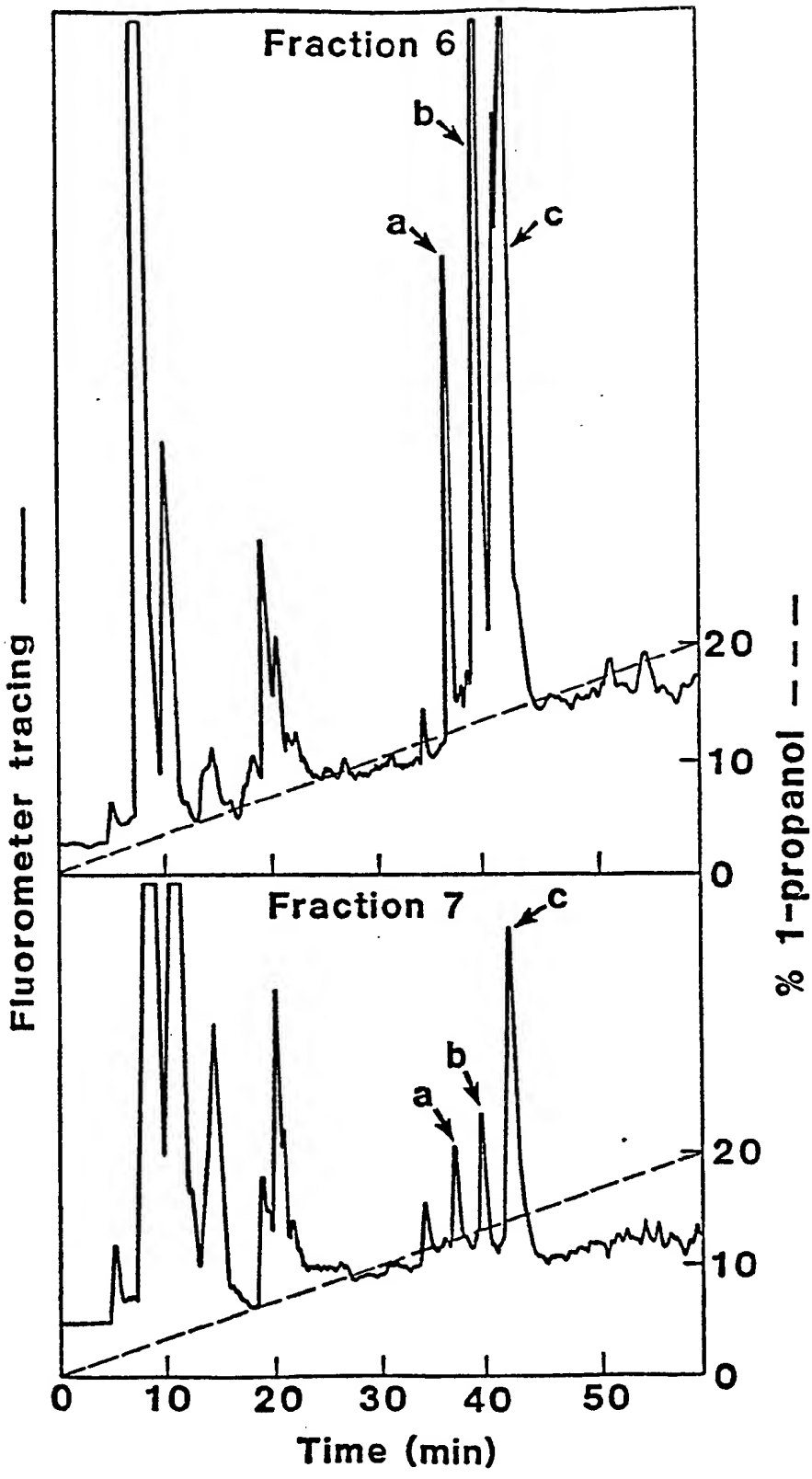

Figure 2

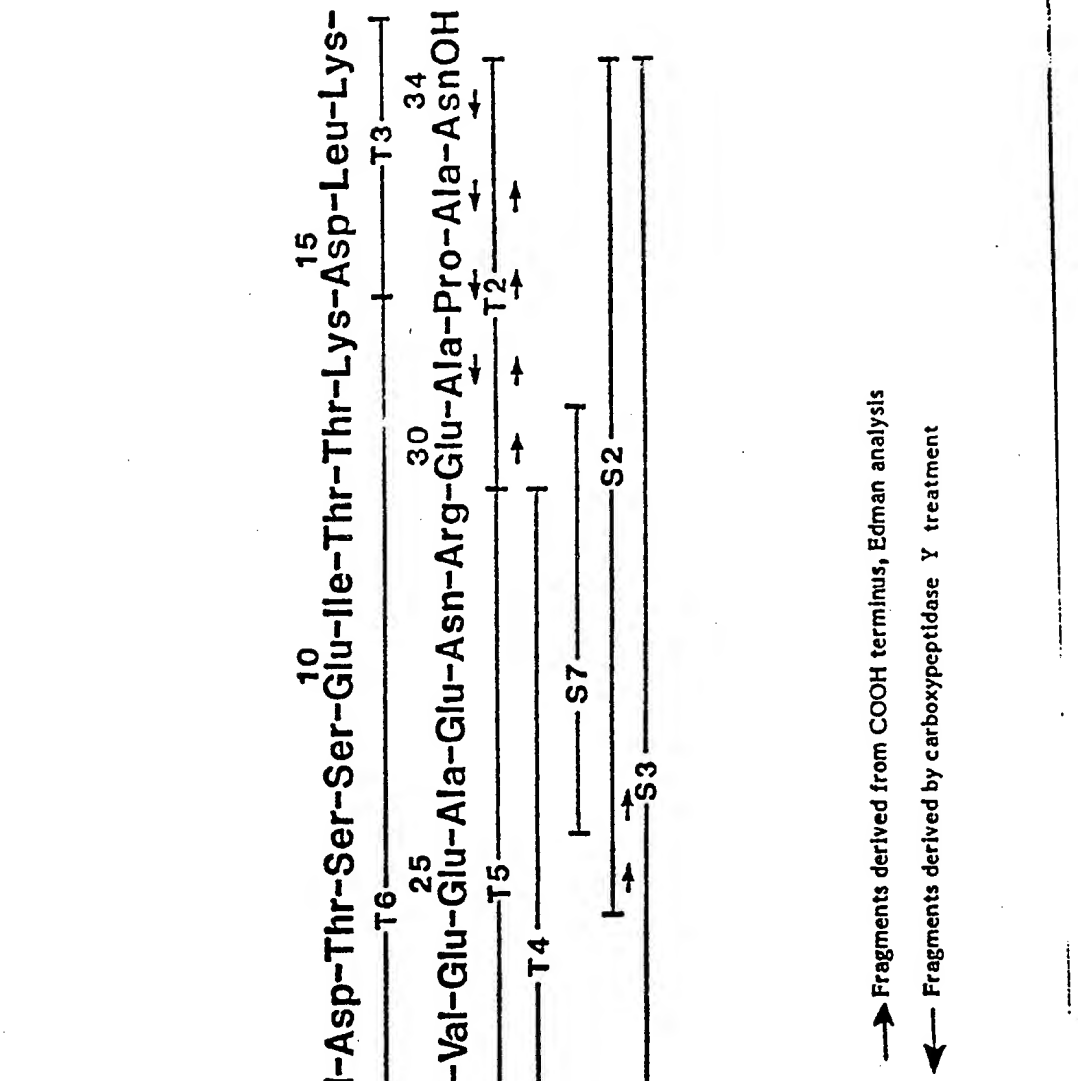

Figure 1

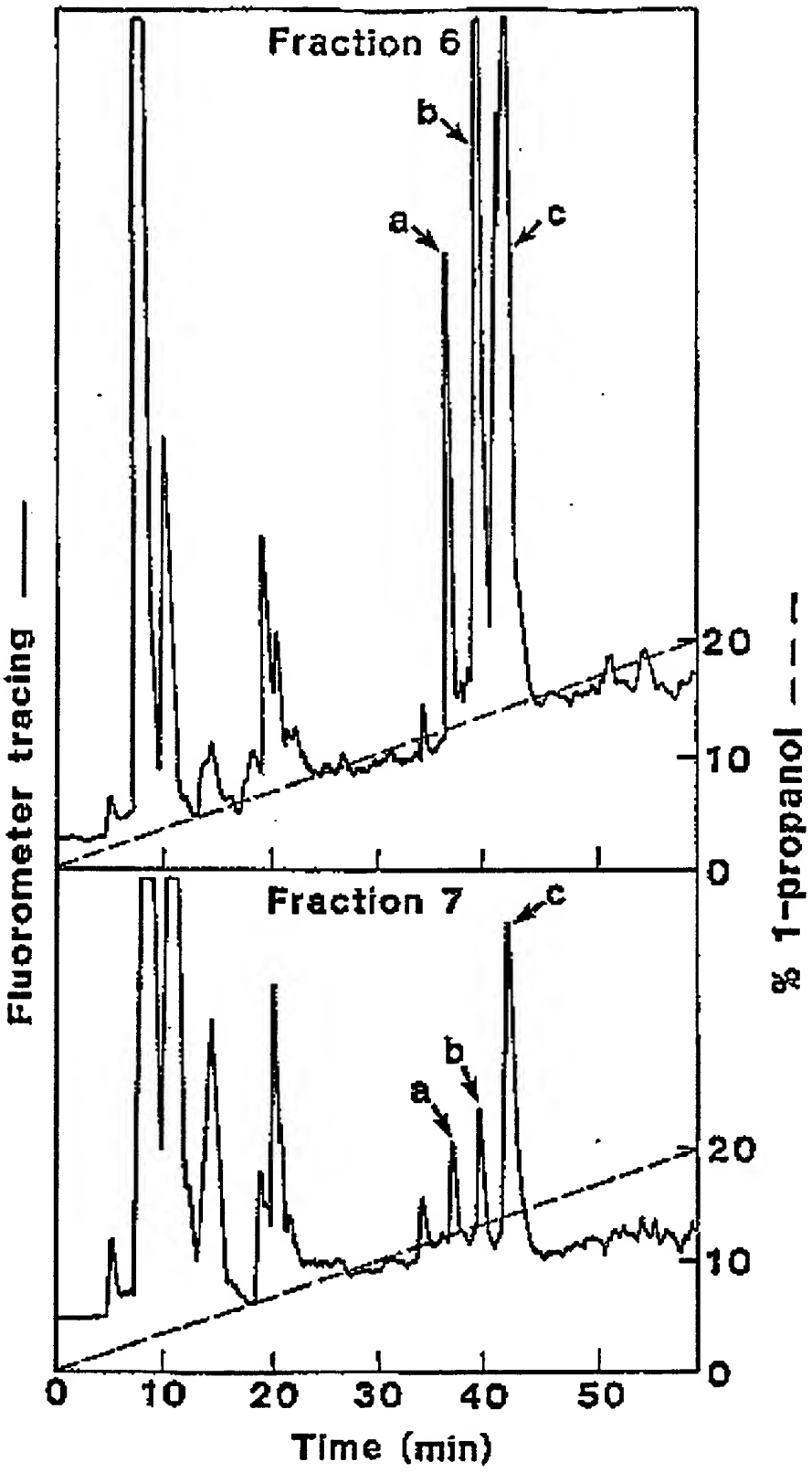

Figure 2